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Glucuronic Acid Rich Kombucha-fermented Pomegranate Juice

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Abstract

This study is the first report using tea fungus "kombucha" to ferment natural pomegranate juice to produce a fermented beverage with high content of glucuronic acid, as a human health beneficial component. We profited the acetic acid bacteria and yeasts symbiotic layer, which is well known in producing pharmaceutical beverages with considerable released organic acids such as glucuronic acid. Also, we used the natural pomegranate juice with high amount of carbohydrate and acid, as a favourable substrate for the fermentation process. The yield of glucuronic acid production was monitored by cultivating natural pomegranate juices under the 17 optimized-combinations of three distinct sucrose concentrations, fermentation temperatures, and processing time. The combinations were designated by applying the statistical response surface methodology method. The maximum amount of glucuronic acid 17.074g/l determined in the media with 8g/l supplementary sucrose after 14 days fermentation at 37℃, using high-performance liquid chromatography. Along with glucuronic acid production, effect of the three factors - sugar concentration, processing temperature and time - was also examined on changes of five physical and chemical properties of the fermented pomegranate juices, including; pH value, remained sucrose and reducing sugar content, kombucha layer biomass, and total acidity. Within 14-day fermentation process, the pH values showed decrease, the layers' mass presented considerable increase, and the total acid content increased in the beverages. Overall, obtained data suggested that natural pomegranate juice can be a potential candidate for further development as a functional beverage to support the maximum human daily intake of glucuronic acid (45mg for a 70kg adult).

Keywords: acidity, fermentation, glucuronic acid, high-performance liquid chromatography, kombucha, pomegranate juice, response surface methodology, sucrose, pH value

1. Introduction

Since 1914, many scientists have stated the healing effect of fermented tea fungus beverages on numerous human diseases, such as inflammation of tonsils, colon colitis, and small intestine as well as risks of arteries walls, blood pressure, structure sclerotic changes, and catarrhal angina (Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014; Dufresne & Farnworth, 2000; Hartmann, Burleson, Holmes, & Geist, 2000; Barbancik, 1958). The health benefits of the beverages have been mostly established that are related to the significant amounts of formed organic acid through fermentation process, such as glucuronic acid (Jayabalan et al., 2014; Vına, Semjonovs, Linde, & Deninxa, 2014). Participation of acetic acid bacteria and yeasts through the fermentation process qualify the kombucha layer in synthesizing cellulose along splitting sucrose into glucose, fructose, and ethanol to produce glucuronic acid (Teoh, Heard, & Cox, 2004; Greenwalt, Steinkraus, & Ledford, 2000).

Besides the fermented kombucha beverages, a natural pomegranate juice has also been considered as a functional beverage because of its enormous health effects especially on atherosclerosis and cardiovascular diseases (Vına et al., 2014; Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2010). It is well-known that its nutritional value is due to having the substantial content of bioactive components such as phenol-carboxylic

acids, anthoxanthins like flavonoids and anthocyanins, astringent-polyphenolic compounds such as tannins, and antioxidants (Viuda-Martos et al., 2010). Moreover, due to high carbohydrates content and acidity, it can be considered as a favourable substrate for fermentation process to produce organic acids, especially glucuronic acid (Kazakos et al., 2016). Furthermore, Mousavi et al. (2013 & 2011) emphasized the fermentation suitability of pomegranate juice, as a functional beverage.

Previous studies on pomegranate juice fermentation, as a single or mixed substrate, using kefir grains (Kazakos et al., 2016), *Saccharomyces cerevisiae* yeast strains (Berenguer et al., 2016), and lactic acid bacteria (*Lactobacillus acidophilus* and *plantarum*) (Mousavi et al., 2013; Filannino et al., 2013) have been reported. However, so far, no analyses considered using kombucha symbiosis layer to ferment pomegranate juice to evaluate the functional properties of produced fermented beverages (in terms of glucuronic acid content) has been reported. As former studies on kombucha fermentation revealed, the basic biochemistry of beverage components can be varied due to variability of sugar contents, incubation time periods, and influenced temperatures. Thereby, in the present study, we aimed to investigate the potential of the glucuronic acid production through pomegranate juice fermentation using kombucha culture, accompanied with measurements of the changes in pH value, amount of final sucrose, reducing sugar, total acidity, and layer biomass within the applied three treatment variables; cultivation sucrose content, temperature, and time, each in three levels. To not only analyse the factors stimulate glucuronic acid production, but also determine the optimum relation between the elements, statistical response surface methodology (RSM) method was applied. The massive total 27 multiple-factors experiments of the three elements, each with three levels, reduced to the 17 optimized-experiments in monitoring glucuronic acid production as well as other parameters.

Remarkably, the obtained results showed substantial effects of the three factors on glucuronic acid formation as well as final chemical and physical properties of the produced beverages.

2. Materials and Methods

2.1 Pomegranate Juice

The pomegranate juice (PJ) products were supplied from the Takdaneh Agri & Ind. Co., Iran. Before shipping to the laboratory, chemical and physical properties of the PJ products were examined by the company specialists, as shown on Table 1. The three distinct concentrations of sucrose, as shown in Table 2, were dissolved in 1000ml of the PJ products, as culture media, before pouring into the 5000ml-glass jars that had previously sterilized at 121 ℃ for 20 min.

Table 1. Chemical and physical properties of pomegranate juice (PJ) products.

Nutrition value (per 1000 ml)	Total acid (g)	H	Glucuronic acid (g)	Carbohydrates (g)	
Pomegranate juice (PJ)	10	3.5	0.4	Reducing sugars	Sucrose
				5.5	6

2.2 Cultivation of Kombucha Layer

The kombucha layers were collected from the Persian Type Culture Collection, IROST. The previously bacterial floral identification of the culture showed that the most abundant bacterial belong to the genera *Acetobacter,* such as *Acetobacter xylinum* and *A. aceti*, as well as *Gluconobacter*. In addition, *Saccharomyces*, *Koleckera*, *Pichia*, and S*chizosaccharomyces* were also recognized as the yeast species in the provided kombucha layers. Next, the sucrose sweetened culture media were inoculated with 15% (w/v) kombucha biomass that had been aseptically preserved in the PJ products at 2 ℃ temperature for 48hrs, before cultivation. Finally, the jars were properly sealed with a sterilized mesh cloth (Figure 1) to maintain fermentation process under an aerobic atmosphere, free of defects or debris, and inside the incubators at the three distinct temperatures, as shown in Table 2. Each jar was sampled only at the three-time points, shown in Table 2, to avoid potential contamination.

Figure 1. An incubator with the sterilized-mesh sealed jars

2.3 pH Value

The pH values were measured using an electronic pH meter (Metrohm model 827) calibrated at the pH 4 and 7, at the room temperature. Three replicate measurements were performed.

2.4 Reducing Sugars and Remained Sucrose Content

The reducing sugars and remained sucrose, at the three sampling time points (Table 2), were determined using the Lane-Eynon general volumetric method. Three replicate measurements were also performed.

2.5 Preparation of Glucuronic Acid Standard Solutions

Apparent concentration 20g/l of the HPLC-grade glucuronic acid, provided from the Fluka Chemical AG. (Industriestrasse 25, CH-9741 Buchs Switzerland), as the primary stock standard solution was prepared by dissolving proper amount of the solid powder in deionized water. Calibration standards for the glucuronic acid analysis were prepared with blank deionized water, resulting in concentrations of 3, 5, 15g/l. The glucuronic acid concentration for each individual sample was derived by comparison with the standard calibration curve (Figure 2).

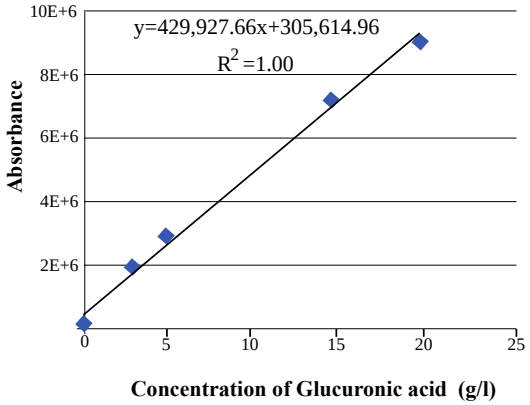

Figure 2. Standard calibration curve of the HPLC-grade glucuronic acid

2.6 High-performance Liquid Chromatography (HPLC) Analysis of Glucuronic Acid Content

The HPLC samples preparation were performed according to the described method by Jayabalan et al. (2007) with some modification. In brief: 20ml of the fermented beverages were centrifuged at 10,000rpm (RCF approx. 14,000×g) within 10min at 8℃. Next, supernatant was diluted in 1/10 using a volumetric flask of 10ml filled with 1/2ml of supernatant and redistilled water. The obtained extract directly passed through the HPLC millipore filter (0.45 µ) vials. A 20µl filtrate was injected to a reverse-phase-chromatography system equipped with a Nucleocil C-18 column (4mm ID ×250mm, 5µm), a single pump Bischoff, and a UV detector. The 50mM sodium dihydrogen phosphate at pH~2.58 was used as a mobile phase. The flow rate 1.0ml/min was maintained at ambient temperature. Detection was carried out at 210nm. Obtained peaks were chronicled on the standard HPLC curve (Figure 2) and multiplied by the dilution factors to quantify the acid concentrations. A typical chromatogram of the assay for glucuronic acid at the substrate concentration of 0.53g/l was shown in Figure 3.

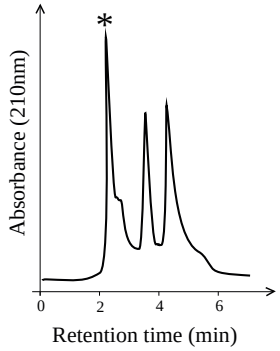

Figure 3. HPLC chromatogram of the glucuronic acid identification in the fermented SPJ with concentration 0.53g/l acid. *; Position of the glucuronic acid peak

2.7 Yield of Biomass

The kombucha biomass was determined using a mass measurement method, described by Malbasa, Loncar, & Djuric, (2008). Briefly, the grown floating cellulosic layer was removed from surface of the fermented beverages (Figure 4), rinsed with distilled water, and dried with filter paper before measuring the weight, at the three time points (Table 2). Three replicate measurements were performed.

Figure 4. The harvested mother and daugther floating cellulosic layers

2.8 Experimental Design

To locate the optimum vicinity of the three-element and three-level effective media factors involved in a glucuronic acid production, including: sucrose content, temperature degree, and time period, the statistical RSM method using the Design Expert Version 6.0.10 (Stat-Ease, USA) was applied, as described by Sayyad, Panda, Javed, & Ali, (2007). The statistical design of the experiments along with nature of the response surface estimation, in the optimum region, resulted in a total number of 17 experiments, given in Table 2.

Table 2. Independent variables and their coded and actual values in the experimental design.

Independent variable	Units	Symbol	Coded level		
			-1	0	1
Temperature	℃	X_1	18	27	37
Time	day	X_2	4	9	14
Sucrose	g/l	X_3	6	8	10

3. Results and Discussion

3.1 pH Value

During the fermentation process, the initial pH value of the PJ products ~3.5 (Table 1) dropped to the lowest value ~2.58, within 14 days, and the highest value ~2.88, on day 4 of the fermentation process (Table 3). This observation could be associated mainly to a remarkable amount of organic acids, especially acetic acids produced through sucrose metabolism and ethanol oxidization via mutual act of kombucha bacteria and yeasts mutual act, within fermentation process (Jayabalan et al., 2016; Jayabalan et al., 2014; Chen & Liu, 2000). This pH reduction was well correlated to the observed increase in the total acidity of the fermented beverages formed within 14-day process of fermentation (Table 3). It also was consistent with the reports from Jayabalan et al. (2016 & 2007). Worth to note that the observed values for pH were slightly lower in compare to the previously published reports of kombucha fermented beverages benefited other different substrates, such as sweetened black tea (Jayabalan, Marimuthu, & Swaminathan, 2007), sweetened sour cherry juice (Yavari, Assadi, Moghadam, & Larijani, 2011), sweetened grape juice (Yavari, Assadi, Moghadam, & Larijani, 2010), and even cheese whey (Belloso-Morales, & Hernández-Sánchez, 2003). However, it was in line with the previous studies presented on apple tea wine by Kumar & Joshi (2016), and on red grape juice by Ayed et al., (2017). The surface plot of the pH value (as a function of the two independent variables; fermentation time (X2) and temperature (X1)), at the sucrose content (X3) 8g/l, were shown on Figure 5-a.

3.2 Total Acidity

The total acid content of the fermented beverages was changed from the initial amount 10g/l in PJ products (Table 1) to the range value 4.2-26.6g/l at the end of the fermentation process (Table 3). As recently determined, various type of acids, predominantly acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, malonic, oxalic, succinic, pyruvic, and usnic acids can be produced in kombucha beverages during fermentation process (Jayabalan et al., 2016). Thus, while we only investigated the changes in glucuronic acid content during

fermentation process, the continuously increase in total acidity of the beverages implied the endless production of tremendous amount of acids within 14-day fermentation process (Table 3). The surface plot analysis of total acidity content in beverages presented in Figure 1-b, as a function of the two independent variables; fermentation time (X2) and temperature (X1), at sucrose content (X3) 8g/l.

It is worth noting that the total acidity content of the fermented beverages varied according to the time period of the process (Table 3), which is in accordance with the previous findings (Yavari et al., 2011; Yavari et al., 2010; Jayabalan et al., 2007). However, kumar et al. (2016) indicated that the fermentation period beyond 10 days may cause the high acidity level with harmful potentiality to human body in direct intake. Therefore, further studies might be aimed to carry out the fermentation process to reduce the acidity of fermented beverages, while sustaining the high-level production of glucuronic acid and other bioactive metabolites.

3.3 Glucuronic Acid Content

Glucuronic acid content of the unfermented and unsweetened PJ products ~4.32g/l (Table 1), showed an extensive variation, at the range of ~0.42-17.07g/l, in the final sweetened and fermented beverages during 14-day fermentation process (Table 3). Previous studies showed that the maximum amount of glucuronic acid ~2.3g/l produced in 12-day fermented beverages, using black tea as a substrate (Jayabalan et al., 2007), while our study presented the maximum amount of glucuronic acid ~17.07g/l for 14-day fermented beverages (Table 3). Considerable variations in glucuronic acid production observed when the PJ products were subjected to the various combination of temperature and time within various sucrose content (Table 3). Thereby, in our study, the significant increase in glucuronic acid content of the beverages suggested the incredible capability of the sweetened pomegranate, as a substrate, and the optimized fermentation conditions, shown in Table 2, in yielding the high amount of glucuronic acid. Remarkably, the yeasts of the kombucha culture are able to hydrolyze the media sucrose into glucose, which is transferred and consequently oxidized at sixth carbon by acetic acid bacteria of the layer to produce glucuronic acid (Jayabalan et al., 2016; & Jayabalan et al., 2007). Hence, the continuously increase in glucuronic acid content observed during fermentation process could obviously supposed the proper relationship between the acid bacteria, *A. xylinum* and *A. aceti*, and the *Saccharomyces*, *Koleckera*, *Pichia*, and S*chizosaccharomyces* yeasts of the kombucha layer in sweetened pomegranate substrate under the fermentation process condition (Table 2). Furthermore, our observation was someway consistent with the study from Nguyen et al. (2015) that indicated the highly effectiveness of removing unwanted microbial strains of the kombucha culture in producing glucuronic acid to over 17.5mg/l, in compared to the traditional kombucha.

Table 3. Box–Behnken design (BBD) matrix for three factors (Temp, Time, and Sucrose) along with the obtained data of six responses (glucuronic acid, remained sucrose, reducing sugar, total acidity, biomass, and pH) for PJ products fermentation process. The PJ products were fermented and sampled as described in ‘‘Materials and Methods’’. The data were expressed as means ±SD of three replicates

Temp	Time	Sucrose	Glucuronic acid	Remained sucrose	Reducing sugar	Total acidity	Biomass	pH
℃	day	g/l	g/l	g/l	g/l	g/l	g	_
18	4	8	0.53+0.01	6.11+0.01	2.2+0.04	4.7+0.05	53.72+0.02	2.88+0.01
18	9	10	1.21+0.04	5.04+0.02	0.69+0.01	9.4+0.01	112.0+0.05	2.8+0.01
18	9	6	0.42+0.04	5.64+0.01	0.76+0.01	9.4+0.02	81.38+0.04	2.83+0.01
18	14	8	6.2+0.04	5.68+0.01	0.9+0.05	15.2+0.02	106.7+0.05	2.76+0.01
27	4	6	0.43+0.03	2.04+0.01	1.8+0.02	4.2+0.03	128.8+0.01	2.87+0.02
27	4	10	0.73+0.01	9.01+0.01	1.5+0.03	7.1+0.01	122.3+0.02	2.84+0.02
27	9	8	2.45+0.01	7.51+0.02	0.67+0.01	15.6+0.02	104.0+0.01	2.8+0.02
27	9	8	2.37+0.02	5.92+0.04	0.48+0.04	8.7+0.03	130.8+0.01	2.82+0.01
27	9	8	3.22+0.01	5.63+0.03	0.55+0.01	13.1+0.02	111.6+0.02	2.8+0.02
27	9	8	2.5+0.03	4.14+0.02	0.64+0.05	10.3+0.02	128.6+0.01	2.82+0.01
27	9	8	2.95+0.01	7.85+0.02	0.55+0.02	13.2+0.01	115.4+0.01	2.81+0.01
27	14	6	3.53+0.02	4.11+0.03	0.98+0.01	12.4+0.01	286.4+0.04	2.65+0.02
27	14	10	6.4+0.04	5.01+0.01	0.51+0.02	14.8+0.01	294.3+0.01	2.69+0.02
37	4	8	5.62+0.03	3.17+0.03	1.31+0.02	8.9+0.04	28.73+0.01	2.79+0.01
37	9	10	5.78+0.04	4.85+0.01	1.1+0.05	14.4+0.05	213.8+0.05	2.71+0.01
37	9	6	5.67+0.05	4.01+0.02	1.14+0.06	11.4+0.03	179.5+0.02	2.72+0.01
37	14	8	17.07+0.0	1.45+0.03	0.38+0.02	26.6+0.04	280.6+0.05	2.58+0.01

3.4 Reducing Sugar and Remained Sucrose

The PJ products were sweetened with three different concentrations of sucrose, as shown in Table 2. Initial content of reducing sugars 5.5g/l and remained sucrose 6g/l for the PJ products (Table 1) showed changes to ~0.38-2.2g/l and 1.45-9.01g/l range value, respectively. These changes accompanied with increase in the total acidity of the final fermented beverages ~4.2-26.6g/l within 14 days of the fermentation process, as shown in Table 3. During kombucha fermentation, enzymes of the yeasts, such as invertase and sucrase, assist culture to hydrolyze the media sugar, sucrose, into mixture of the reducing sugars glucose, and fructose, which are prerequisite component for ethanol, and consequently organic acids production throughout the kombucha aerobic fermentation process (Jayabalan et al., 2014; Chen & Liu, 2000). Thereby, the high content of the total acidity and glucuronic acid recommended highly action of the kombucha culture microbes on media sugar. Moreover, elevated amount of glucuronic acid along with contrary changes in amount of the remained sucrose and reducing sugars, decreased and increased respectively, indicated that the added sucrose to the media was continuously hydrolysed to the reducing sugars to produce organic acids. This observation was consistent with the recent results from Jayabalan et al. (2016). Furthermore, small varied range of the remained sucrose among the beverages (Table 3 & Figure 5-d), indicated that the sucrose consumption rates through fermentation process were almost the same. However, the lowest amount of remained sucrose ~1.45g/l for the media with the highest amount of produced glucuronic acid might suggested that the involved yeasts and bacteria, in the kombucha fermentation process, were probably more active and durable during 14 days of fermentation.

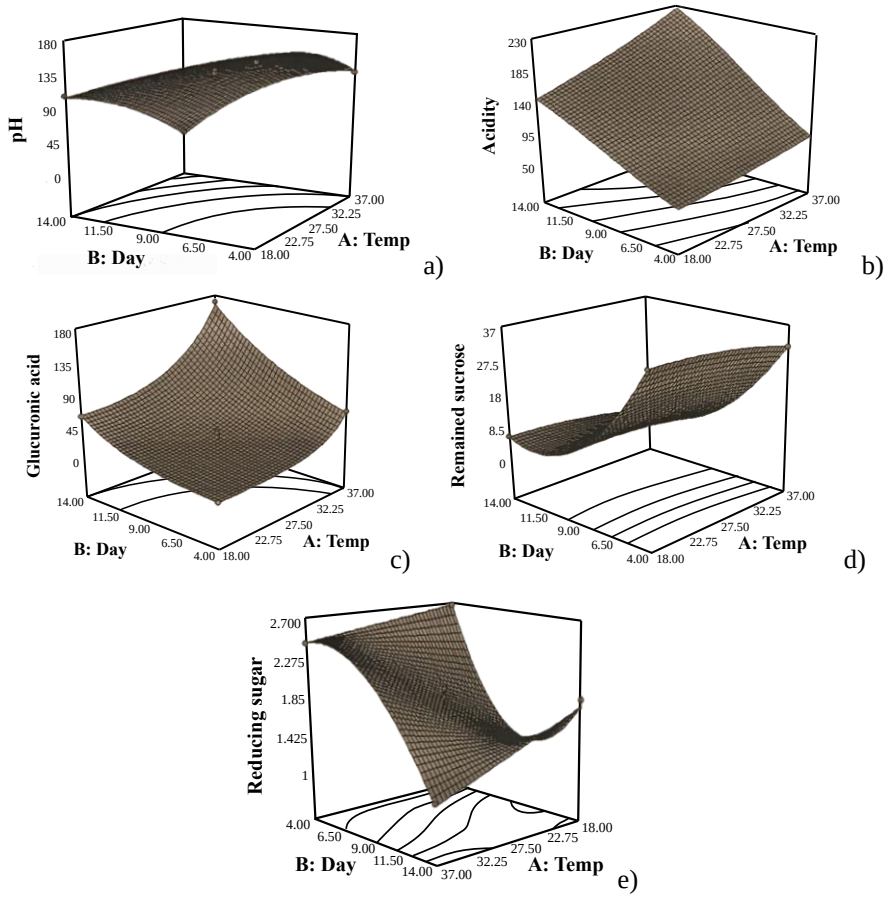

Figure 5. Surface plots of the pH value (a); acidity (b); glucuronic acid (c); remained sucrose (d) and reducing sugar (e) changes of the PJ products fermented as a function of temperature (A: Temp) and fermentation time (B: Day). The PJ products were fermented and sampled as described in ‘‘Materials and Methods’’

3.5 Yield of Biomass

The smallest layer mass ~28.73g, and the largest mass ~294.3g were obtained on 4 and 14 days after fermentation process, respectively (Table 3). Therefore, it was obvious that the fermentation period had a tremendous effect on the kombucha biomass yield. Furthermore, not only time period of the process, but also

reducing sugar content such as glucose, produced from the decomposed sucrose, showed profound influence on cellulose and hemi-cellulose production (Bauer‐Petrovska & Petrushevska‐Tozi, 2000). While later revealed by keshk & Sameshima, (2006) that reducing sugar, as a carbon source, can have a relative contradictory function in acid and biomass production, our observation was not consistent with their arguments.

3.6 Sensory Characteristics

When the symbiosis culture placed in the jars containing the PJ products, the CO_2, generated through the fermentation process by the yeasts, accumulated in the interface between the the PJ products media and the kombucha layer to create an anaerobic atmosphere to allow the fermentation process to fulfilled (Malbasa et al., 2008; Jayabalan et al., 2007). During fermentation process, the PJ products turned into the light red colored with a sour taste, little sparkling surface, and acidified smell beverages. Evaluation tests exhibited that the acidity tastes of the beverages, even on 14 days after fermentation process, were acceptable from the sensory characteristics viewpoint (Table 4). The endpoint sensory analysis of the fermentation process, on day 14, showed the considerable increase in both sugar and alcohol contents, while the acidic taste moves from sweet to remarkable sour (Table 4).

Table 4. Sensory evaluation of the fermented PJ products. The fermented beverages were fermented and sampled as described in ‘‘Materials and Methods’’. Data extracted from a team of 16 evaluators in three replicates.

Temp	Time	Sucrose	CO_2 content	Sugar taste	Acidic taste	Alcoholic taste	Ferment smell	Transparency
18	4	8	3	2	3	2	2	5
18	9	10	2	2	3	2	1	4
18	9	6	3	1	2	1	2	4
18	14	8	3	3	3	1	1	5
27	4	6	4	1	3	2	3	3
27	4	10	3	1	4	2	5	2
27	9	8	4	3	1	1	2	3
27	9	8	2	2	2	5	4	4
27	9	8	3	1	1	1	3	3
27	9	8	2	2	1	1	4	4
27	9	8	2	2	2	4	2	4
27	14	6	4	2	3	2	3	4
27	14	10	3	2	2	2	4	3
37	4	8	4	3	2	5	3	1
37	9	10	3	2	1	5	4	1
37	9	6	1	2	1	5	3	1
37	14	8	4	3	3	5	3	1

Note: The sensory evaluation criteria; Scores 1-5 were describing dislike extremely, disliked moderately, neither liked nor disliked, liked moderately, and liked extremely, respectively.

4. Conclusion

In the current study, the entire obtained data suggested that among the various traditional substrates used to produce the glucuronic acid, pomegranate juice presented as the desired substrate to produce the considerable amount of glucuronic acid with the highest value of 17.07g/l. Also, high production of the glucuronic acid along biomass yield suggested the usefulness of the pomegranate juice, as a cost-effective substrate source, in high-yield industrial production that indicated as a key issue by Hong & Qiu, (2008).

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